

University of Groningen

## Circadian regulation of the light input pathway in *Neurospora crassa*

Merrow, Martha; Franchi, Lisa; Dragovic, Zdravko; Görl, Margit; Johnson, Judy; Brunner, Michael; Macino, Giuseppe; Roenneberg, Till

*Published in:*  
The EMBO Journal

**IMPORTANT NOTE:** You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2001

[Link to publication in University of Groningen/UMCG research database](#)

### *Citation for published version (APA):*

Merrow, M., Franchi, L., Dragovic, Z., Görl, M., Johnson, J., Brunner, M., Macino, G., & Roenneberg, T. (2001). Circadian regulation of the light input pathway in *Neurospora crassa*. *The EMBO Journal*, 20(3), 307-315.

### Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

### Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

# Circadian regulation of the light input pathway in *Neurospora crassa*

Martha Merrow, Lisa Franchi<sup>1,2</sup>,  
Zdravko Dragovic, Margit Görl<sup>3</sup>,  
Judy Johnson<sup>4</sup>, Michael Brunner<sup>3</sup>,  
Giuseppe Macino<sup>2,5</sup> and Till Roenneberg<sup>5</sup>

Institute for Medical Psychology, <sup>3</sup>Institute for Physiological Chemistry and <sup>4</sup>Institute for Immunology, University of Munich, D-80336 Munich, Germany. <sup>1</sup>Department of Biology, New York University, New York, NY, USA and <sup>2</sup>Dipartimento di Biotecnologie Cellulari ed Ematologia, Università di Roma, 00185 Rome, Italy

<sup>5</sup>Corresponding authors

e-mail: till.roenneberg@imp.med.uni-muenchen.de or  
Macino@Bce.med.uniroma1.it

**FREQUENCY (FRQ) is a critical element of the circadian system of *Neurospora*. The *white collar* genes are important both for light reception and circadian function. We show that the responsiveness of the light input pathway is circadianly regulated. This circadian modulation extends to light-inducible components and functions that are not rhythmic themselves in constant conditions. FRQ interacts genetically and physically with WHITE COLLAR-1, and physically with WHITE COLLAR-2. These findings begin to address how components of the circadian system interact with basic cellular functions, in this case with sensory transduction.**

**Keywords:** circadian/frequency/light/*Neurospora*/white collar

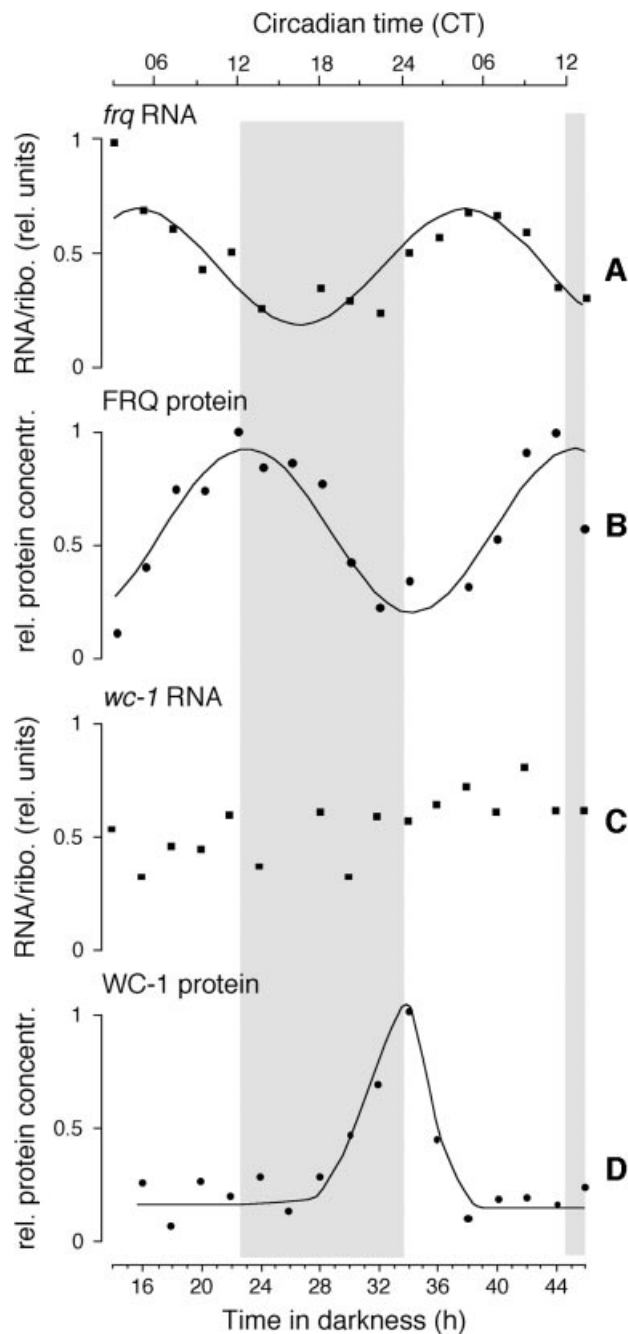
## Introduction

Circadian systems coordinate the temporal program of organisms in all phyla to accommodate and anticipate the daily changes of the environment (Pittendrigh, 1993). Experimentally, circadian rhythms are characterized by their self-sustained, ~24 h oscillation in constant conditions. Rhythms are entrained (in nature to 24 h) by appropriate environmental signals (*zeitgeber*), of which light is the most studied (Roenneberg and Foster, 1997). An intact circadian system includes input pathways, a mechanism that generates rhythmicity (rhythm generator) and outputs. Physiological experiments show that light input pathways (LIPs) to the rhythm generator may themselves be under circadian control. For example, in the marine unicell *Gonyaulax*, one of the circadian LIPs is only active during the subjective night (for definition of subjective day and night, see legend to Figure 1; Roenneberg and Taylor, 1994; Roenneberg and Deng, 1997). There are also indications that light receptors of circadian systems are rhythmic, e.g. cryptochrome in *Drosophila* (Emery *et al.*, 1998) or phytochrome B in plants (Bognar *et al.*, 1999).

Thus, circadian light input is probably not a straightforward transduction of signals to the rhythm generator, but rather an active, circadianly regulated mechanism. In principle, input pathways can influence circadian rhythmicity by responding both to external *zeitgebers* and to the endogenous circadian system. Modeling shows that mutations in components of rhythmic input pathways change period as determined in constant conditions and contribute to self-sustainment. These theoretical results (Roenneberg and Merrow, 1998, 1999) are in accordance with reports showing that mutations in genes encoding input elements can change period or even result in arrhythmicity in constant conditions (Millar *et al.*, 1995; Somers *et al.*, 1998; Iwasaki *et al.*, 2000). Either of these observations would also be consistent with mutations in components that are central to the rhythm generator.

In all molecular/genetic model systems, a negative feedback loop is essential for self-sustained circadian rhythmicity in constant conditions and is generally considered to be central to the rhythm generator. These loops involve expression of genes to proteins (transcription, translation and modification, e.g. phosphorylation) which, in turn, inhibit their own expression. Such a transcription/translation feedback loop is also necessary for self-sustainment of circadian rhythms in *Neurospora*, involving the genes *frequency* (*frq*), *white collar-1* (*wc-1*) and *white collar-2* (*wc-2*) as central players (Dunlap, 1999). Initially, *wc-1* and *wc-2* were identified in screens for lack of light reception (Harding and Turner, 1981; Degli-Innocenti and Russo, 1984). They are both light-inducible, DNA-binding, putative transcription factors (Ballario *et al.*, 1996; Linden and Macino, 1997), and WC-1 shares similarity with a class of proteins from diverse species that actively process electrons or photons (Huala *et al.*, 1997). Also, WC-1 and WC-2 regulate basal levels of *frq* (Crosthwaite *et al.*, 1997), which was identified in screens for circadian period mutants (Feldman and Hoyle, 1973). FRQ negatively regulates its own transcription in constant conditions (Aronson *et al.*, 1994b), providing a mechanistic basis for continuous oscillations (self-sustained rhythmicity). However, circadian properties remain in the absence of the *frq*/FRQ transcription/translation feedback loop. FRQ-deficient strains are capable of circadian entrainment (a special, circadian form of synchronization) in temperature cycles, whereas light entrainment fails, indicating a functional role for FRQ in the LIP (Merrow *et al.*, 1999) and opening up the possibility of additional circadian machinery that functions in the absence of FRQ (see also Lakin-Thomas and Brody, 2000).

Here, we characterize how FRQ is involved in light signal transduction. Two distinct physiological responses to light (conidial banding and carotenogenesis) represent two separate destinations of this light transduction pathway. The former has an absolute requirement for FRQ to



**Fig. 1.** Circadian time course of *frq* and *wc-1* RNA and protein levels. *frq*<sup>+</sup> was grown at 25°C in constant darkness. Samples were harvested at 2 h intervals and *frq* RNA (A), FRQ protein (B), *wc-1* RNA (C) and WC-1 protein (D) levels were measured. Specific RNA signals were normalized to rRNA (RNA/ribo. rel. units) and specific protein signals were normalized to amido black-stained blots (rel. protein concentration). In rhythmic time series (A, B and C), the highest value was set equal to 1; in (C), the average signal was set equal to 0.5. See Materials and methods for curve fits. The initial light to dark transfer of the cells corresponds to dusk and is designated circadian time (CT) 12. See Materials and methods for determination of CT. Gray areas indicate subjective night (i.e. the half of the full circadian cycle that corresponds to darkness in a light:dark cycle); open areas are subjective day.

respond to light, the latter only requires FRQ for circadian regulation and overall magnitude of light induction. We further show genetic and physical interaction of FRQ and

the WC proteins, demonstrating that circadian regulation and light signal transduction are hard wired together.

## Results

### Regulation of LIP components

The components that have been characterized most thoroughly for their early involvement in light responses in *Neurospora* are WC-1 and WC-2. Both proteins are critical for normal circadian rhythmicity; however, *wc-1* mRNA is regulated more robustly by light than *wc-2* (Linden and Macino, 1997). Thus, for questions regarding the relationship between light and circadian regulation, we focused our studies on interactions of *wc-1* and *frq*.

One of the important circadian features of *frq* RNA and protein is their rhythmicity in constant darkness (DD; Figure 1A and B; Aronson *et al.*, 1994b; Garceau *et al.*, 1997). We analyzed *frq* and *wc-1* RNA and protein levels under these conditions. The RNA levels of *wc-1* were variable, but not circadian (Figure 1C; Lee *et al.*, 2000). WC-1 protein levels, however, changed with circadian time (Figure 1D; Lee *et al.*, 2000). The period of the WC-1 oscillation is specific for different circadian period mutants (Lee *et al.*, 2000). The differences for *frq* and *wc-1* RNA and protein profiles in constant conditions show that their regulation is distinct. This is also indicated by the fact that maximum WC-1 protein levels coincide with the FRQ minimum (Figure 1B and D; Lee *et al.*, 2000).

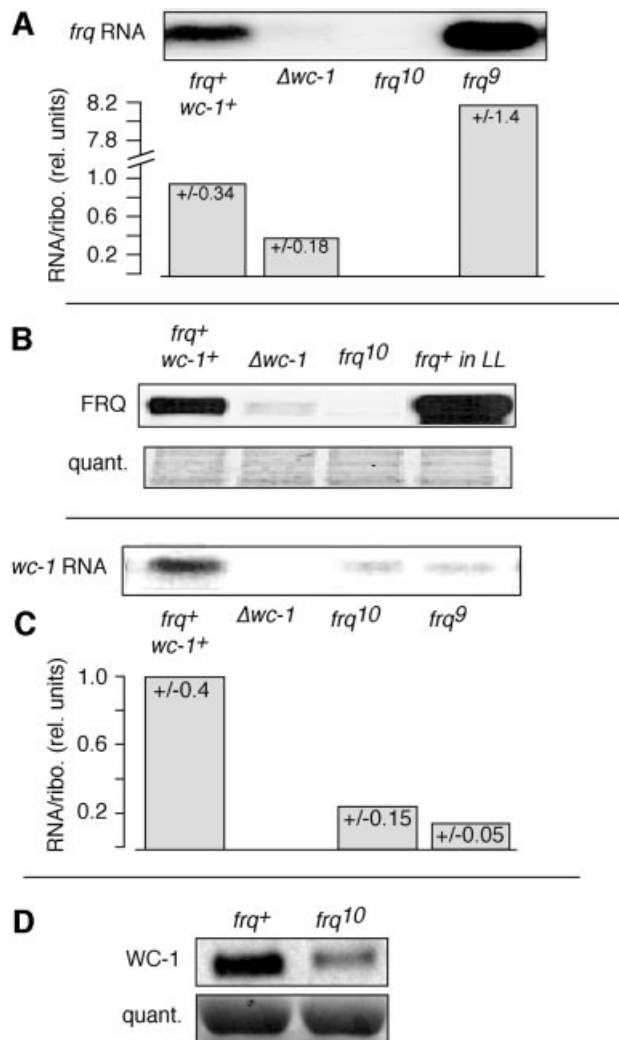
### Regulation of FRQ and WC-1 is interdependent

Basal *frq*/FRQ levels are low in *wc-1* and *wc-2* mutants (Crosthwaite *et al.*, 1997). Here, these levels were determined in a  $\Delta wc-1$  strain (RIPed to a functional knockout; see Materials and methods and Talora *et al.*, 1999), confirming that *frq* RNA and FRQ protein (Figure 2A and B) levels are substantially lower in the  $\Delta wc-1$  than in a *wc-1*<sup>+</sup> strain. These observations suggest that regulation of *frq* lies downstream of WC-1.

Recently, the activators of genes in the *Drosophila* circadian transcription/translation feedback loop were shown to depend on 'downstream' gene products for their expression (Bae *et al.*, 1998; Glossop *et al.*, 1999). A comparative analysis would suggest that this might be a common regulatory mechanism in circadian molecular networks. The rhythmicity of WC-1 (Figure 1D; Lee *et al.*, 2000) suggests a similar interactive network between FRQ and the *white collar* gene products. We therefore determined *wc-1* RNA levels in *frq*<sup>10</sup> (a *frq* null strain, see Materials and methods for description). Basal RNA levels were lower than those observed in *frq*<sup>+</sup> grown in DD (Figure 2C; in contrast to Lee *et al.*, 2000), and WC-1 protein levels were reduced similarly (Figure 2D; and as in Lee *et al.*, 2000). Thus, at least under some conditions (see Discussion), FRQ apparently is required to maintain basal levels of *wc-1* RNA and protein in DD.

### FRQ deficiency and light responsiveness

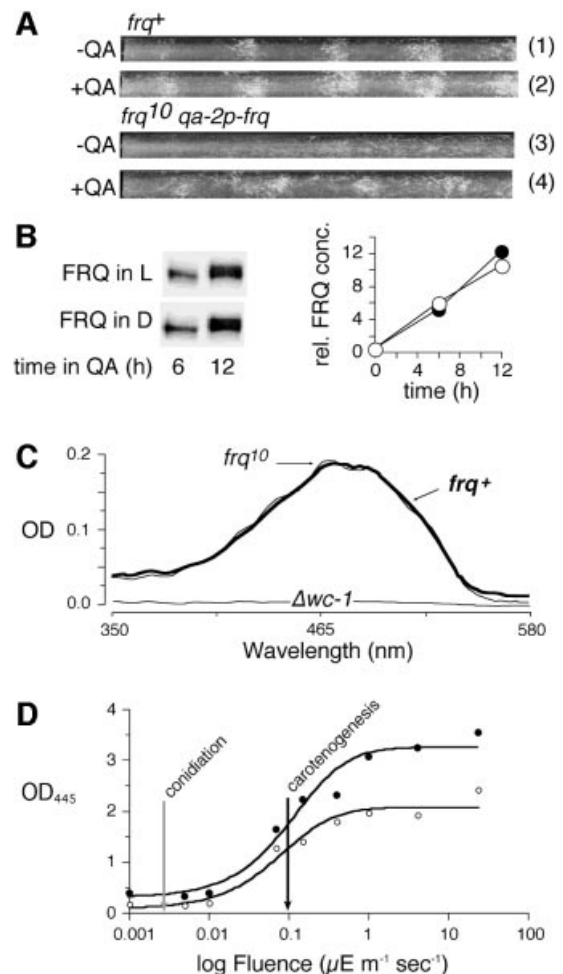
We probed the functional consequences of *frq* deficiency, and the resultant depressed WC-1 levels, on light-induced physiology by investigating several light-inducible outputs: conidial band formation, carotenogenesis and expression of specific RNAs. Figure 3A shows light regulation of conidial banding in a wild-type strain (tubes 1 and 2). As



**Fig. 2.** Regulation of *frq* and *wc-1*. Mycelial pads from *frq*<sup>+</sup>, *Δwc-1*, *frq*<sup>10</sup> and *frq*<sup>9</sup> were incubated in darkness for 28 h and then RNA and protein extracts were prepared. (A) *frq* RNA is reduced in *Δwc-1*. *frq* RNA was detected by northern blotting (upper panel), quantified and normalized to rRNA levels (lower panel). The columns in the graphs represent averages of four experiments (standard deviations are given at the top of each bar). The controls *frq*<sup>10</sup> and *frq*<sup>9</sup> show no *frq* or elevated RNA, respectively, as expected. (B) FRQ protein is reduced in *Δwc-1*. FRQ protein was detected by western blotting. To control for equal loading of the gel, a portion of the blot was stained with amido black (lower panel). The controls *frq*<sup>10</sup> and *frq*<sup>+</sup>LL (constant light) show no FRQ protein or elevated levels, respectively. (C) *wc-1* RNA is reduced in *frq*<sup>10</sup> and *frq*<sup>9</sup>. RNA was analyzed as described above. (D) WC-1 protein levels are reduced in *frq*<sup>10</sup> as shown by western blotting. The amido black-stained membrane indicated equal loading (lower panel).

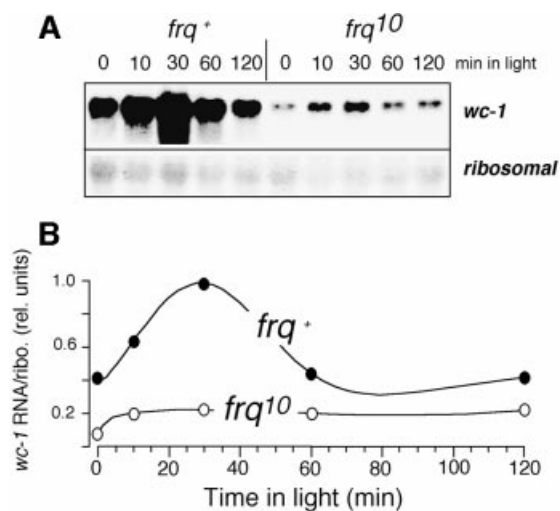
previously reported, *frq* null strains do not respond to light:dark (LD) cycles with succinct formation of conidial bands or their synchronization (Figure 3A, tube 3; Chang and Nakashima, 1997; Merrow *et al.*, 1999; Lakin-Thomas and Brody, 2000; Roenneberg and Merrow, 2001). In contrast, temperature cycles entrain conidial band formation in *frq*<sup>9</sup> (Merrow *et al.*, 1999).

To clarify whether FRQ, as such, is required for light-induced conidial band formation or whether this function depends on the intact negative feedback regulation of the *frq*/FRQ loop, we constitutively expressed FRQ in *frq*<sup>10</sup> (using a *qa-2p-frq* fusion construct similar to that of



**Fig. 3.** A bifurcated light input pathway: FRQ is required for conidiation but not for carotenogenesis. **(A)** FRQ rescues light-regulated conidiation in *frq<sup>10</sup>*. Race tubes were inoculated with *frq<sup>+</sup>* (*bd*) or *frq<sup>10</sup> qa-2p-frq* (*bd frq<sup>10</sup> his3::his3qa-2p-frqhis*), which expresses a His-tagged version of FRQ under the control of the inducible *qa-2* promoter. The race tubes were incubated in 12 h light:12 h dark (LD 12:12) cycles with or without quinic acid (QA). Approximately 4.5 cycles are shown. **(B)** QA-induced expression of FRQ is unaffected by light. *frq<sup>10</sup> qa-2p-frq* was harvested in either the light or dark portion of the third day of an LD 12:12 cycle. At time 0, FRQ expression was induced by QA. Mycelia were harvested after 6 and 12 h and FRQ was analyzed by western blotting (left panel). Signals were quantified by densitometry. Relative expression levels (rel. FRQ conc., right panel) are the average of triplicate samples, normalized for loading based on amido black-stained membranes. **(C)** Induction of carotenoids by light does not require FRQ. Carotenoids were induced in *frq<sup>+</sup>* (thick line), *frq<sup>10</sup>* (thin line) and  $\Delta wdc-1$  with 4  $\mu\text{E}/\text{m}^2/\text{s}$  of light for 5 h. Samples were extracted with hexane and absorption spectra were determined. The *frq<sup>+</sup>* extract was diluted 2-fold relative to the other samples. **(D)** The fluence threshold of carotenogenesis is independent of FRQ. Carotenoids were induced in *frq<sup>+</sup>* (filled circles) and *frq<sup>10</sup>* (open circles) over the indicated range of fluences. Absorption of carotenoids was determined at 445 nm. Values  $>1.0$  were measured as dilutions for accuracy. The black arrow indicates the fluence rate at which half-maximal light induction of carotenoids occurs for both strains. For comparison, the gray arrow indicates the fluence threshold for light-dependent conidial band formation in *frq<sup>+</sup>* (Morrow *et al.*, 1999; Roenneberg and Morrow, 2001).

Aronson *et al.*, 1994b). Although under these conditions this response is not entirely normal, a light-regulated conidiation response was rescued (Figure 3A, tube 4); this strain remains arrhythmic in DD (data not shown; and



**Fig. 4.** Robust light induction of *wc-1* RNA requires FRQ. *frq*<sup>+</sup> and *frq*<sup>10</sup> were incubated for 28 h in the dark and then exposed to 4  $\mu\text{E}/\text{m}^2/\text{s}$  of light. Samples were harvested over 2 h. (A) *wc-1* and rRNAs were detected by northern blot analysis. (B) *wc-1* RNA was quantified and normalized to the amount of rRNA. The maximal signal was set equal to 1. Data are the average of duplicate samples and representative of three experiments.

Aronson *et al.*, 1994b). The timing of conidiation following the light signal (its phase angle) is similar to that for the *frq*<sup>+</sup> strain. The reconstitution of light responsiveness could be mediated by light-induced rhythmicity of FRQ levels, even in the absence of transcriptional regulation. However, when FRQ was induced in the *frq*<sup>10</sup> strain, there was no difference in phosphorylation state (judged by mobility in SDS-PAGE; Figure 3B, left panel) or protein accumulation (right panel) in light versus darkness (Figure 3B). Thus, expression of FRQ is required for light regulation of conidiation, even in the absence of negative feedback of FRQ on *frq*. Given that there is not an obvious qualitative or quantitative difference in FRQ protein induced in light or dark (Figure 3B), it is possible that rhythmicity of the protein is not essential for this response.

Because it was reported previously that the *frq* null strains do show light-induced gene expression (Arpaia *et al.*, 1993, 1995), we investigated light-induced mycelial carotenogenesis (De Fabo *et al.*, 1976). Figure 3C shows that this light response remained qualitatively intact in *frq*<sup>10</sup>: the absorption spectrum of hexane-extracted, light-induced tissue was the same in *frq*<sup>10</sup> and *frq*<sup>+</sup>. However, final carotenoid concentrations were approximately half in *frq*<sup>10</sup> compared with *frq*<sup>+</sup> (the two superimposed spectra represent extracts of different dilutions; see Figure 3C legend). Note that light-dependent carotenoid synthesis is entirely absent in *wc-1* mutants (Harding and Turner, 1981; Linden *et al.*, 1999).

When fluence response curves for light induction of carotenogenesis in *frq*<sup>+</sup> and *frq*<sup>10</sup> were compared, the amplitude of the saturation response in *frq*<sup>10</sup> was about half that of *frq*<sup>+</sup> (Figure 3D). The sensitivity of both strains (fluence rate at half-maximal response, black arrow) was, however, identical. Comparison of the fluence threshold for carotenogenesis with that for light-driven synchronization of conidiation (Figure 3D, gray arrow; Merrow *et al.*,

1999; Roenneberg and Merrow, 2001) suggests two, distinct, light-regulated pathways. Without FRQ, light-regulated conidial band formation is absent even in high light intensity LD cycles, but carotenoids are induced normally based on fluence threshold, although overall accumulation is about half. So, while both branches of this pathway are light blind without WC-1, they are each modulated differently by FRQ (see Figure 7A).

Carotenoid production is the result of a complex, multi-step process. In contrast, one of the earliest detectable events after light exposure of *Neurospora* is induction of *wc-1* RNA, which occurs in <2 min at high light intensities (P.Ballario, unpublished data). Induction of *wc-1* RNA was, therefore, used to monitor early events in light signal reception and transduction, and contrasts the endpoint that carotenogenesis represents. The amplitudes (maximum response relative to baseline) of light-induced RNA in *frq*<sup>+</sup> and *frq*<sup>10</sup> appeared to be similar (Figure 4); however, the peak light-induced *wc-1* levels were low in *frq*<sup>10</sup>, reaching, at most, the basal DD levels of *wc-1* in *frq*<sup>+</sup> (compare with Figure 2C). *al-1* RNA was also induced weakly by light in *frq*<sup>10</sup> (data not shown). *al-1* encodes a downstream enzyme on the carotenogenesis pathway (phytoene dehydrogenase) (Schmidhauser *et al.*, 1990). Given that RNA induction is a relatively rapid and discrete response compared with carotenogenesis, the disparity in strength of carotenoid and RNA induction is not directly comparable.

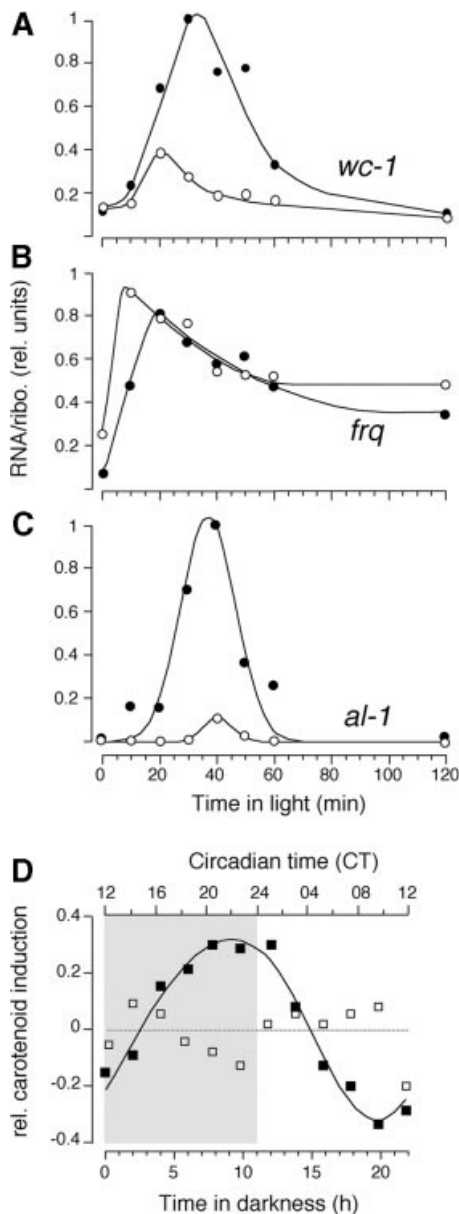
#### Circadian regulation of light responses

Light responses depend on FRQ and WC-1 (Figures 3A, C and D, and 4), and both proteins show a circadian rhythm in abundance (Figure 1B and D). We therefore investigated the physiological light responses described above at different times of the circadian cycle. Time courses measured over 2 h in the middle of the subjective day and night showed large differences in light-induced gene expression. While basal levels of *wc-1* were indistinguishable (see also Figure 1C), the amplitude of the response in the subjective day was less than half when compared with the subjective night (Figure 5A).

*frq* RNA levels depend on WC-1 and WC-2, for both basal expression and rapid and robust light induction (Crosthwaite *et al.*, 1997). In contrast to *wc-1*, *frq* RNA is induced to approximately the same maximal levels at the opposite circadian times (Figure 5B; Crosthwaite *et al.*, 1995). Because *frq* levels are circadian (Figure 1A; Aronson *et al.*, 1994b; Garceau *et al.*, 1997), *frq* is already at different levels at the beginning of light incubation.

Finally, *al-1* light induction resembles the pattern of *wc-1* RNA induction, but the subjective night/day ratio is ~10-fold (Figure 5C). Interestingly, *wc-1* and *al-1* RNA profiles, and to a lesser extent *frq*, show a transiency in prolonged light exposure (Schmidhauser *et al.*, 1990; Arpaia *et al.*, 1995; Linden *et al.*, 1999), resembling classical adaptation responses. RNA levels are down-regulated within 1–2 h and, at least for *wc-1*, remain constant for 10 h of illumination (data not shown). The adaptation profile is apparent at both circadian times that were evaluated.

Lastly, we determined light-induced mycelial carotenogenesis at different circadian times. Light-dependent accumulation of carotenoids was also circadianly regulated, peaking before subjective dawn (notably, the



**Fig. 5.** Light responses are regulated by the circadian system. Time of day-specific light induction of *wc-1* RNA (A), *frq* RNA (B) and *al-1* RNA (C). Mycelial pads were incubated for 16 h (~CT05, unfilled circles) and 27 h (~CT17, filled circles) in the dark and then exposed to light (0.4  $\mu\text{E}/\text{m}^2/\text{s}$ ). After the indicated time periods, RNA was prepared, analyzed by northern blotting and quantified based on rRNA values. The maximal signal was set equal to 1. (D) Light-induced carotenogenesis over the course of a circadian cycle. *frq*<sup>+</sup> (filled squares) and *frq*<sup>10</sup> (open squares) mycelia were transferred from light to dark. After the indicated time in darkness, samples were exposed to light for 5 h to induce carotenogenesis. Carotenoids were extracted with hexane and quantified by absorption at 445 nm. Circadian time of *frq*<sup>+</sup> is shown on top; the gray background indicates subjective night.

circadian modulation of light-induced of carotenogenesis is absent in *frq*<sup>10</sup>, Figure 5D). Thus, circadian regulation of light responsiveness is evident at numerous levels, from some of the earliest known events of light detection to enzymatically regulated, downstream outputs.

**FRQ is physically associated with the WC proteins**  
WC-1 and WC-2 are found in the same complex *in vivo* (Talora *et al.*, 1999). We wondered if the involvement of

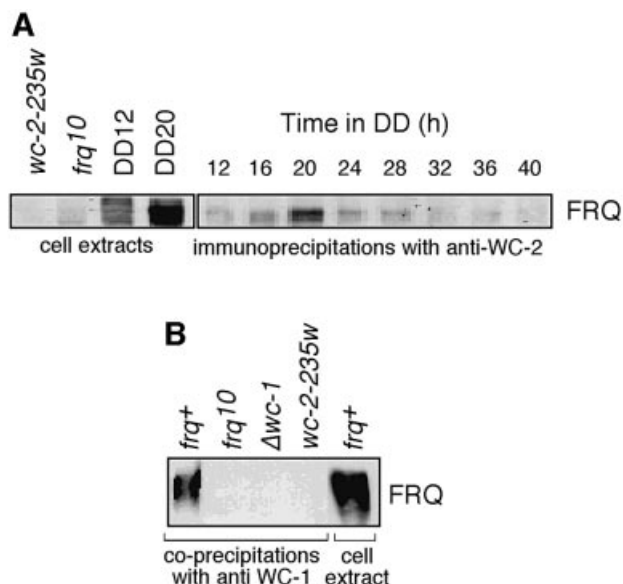
FRQ in light responses could stem from a physical association with either of these proteins. We probed anti-WC-2 immunoprecipitates of cell extracts and found that FRQ binds to WC-2 (Figure 6A). The amount of FRQ in the complex correlated with its circadian accumulation in crude cell extracts (Figure 1B). For reference, FRQ in total cell extracts from *frq*<sup>+</sup> is shown at two circadian times: one harvested at subjective dawn with low levels of highly phosphorylated FRQ, the other at late subjective day with large amounts of less phosphorylated FRQ (Figure 6A, left panel, right lanes). In this experiment, highly phosphorylated FRQ apparently does not participate in complex formation, although in some others it did. Using anti-WC-1, we were also able to immunoprecipitate FRQ from cell extracts of mycelia grown in constant light (LL; Figure 6B). Thus, FRQ is in complexes containing WC-2 and/or WC-1 *in vivo* and WC-2 is found in association with WC-1 (Talora *et al.*, 1999).

## Discussion

We previously observed circadian entrainment by temperature cycles in *Neurospora* strains deficient in FRQ (Morrow *et al.*, 1999). The same strains, however, fail to synchronize to light cycles (Figure 3A; Chang and Nakashima, 1997; Morrow *et al.*, 1999; Lakin-Thomas and Brody, 2000), suggesting that FRQ is required in processing light signals, in addition to controlling essential circadian properties, such as self-sustained rhythmicity in DD. Here, we address several questions about the role of *frq*/FRQ in the LIP and the circadian system of *Neurospora crassa*. What is the nature of the interaction between the WC proteins and their complex (WCC) and FRQ? What is the role of FRQ in light transduction and how does it contribute to entrainment? Figure 7 is a diagrammatic view of the *Neurospora* LIP, specifically with respect to WC-1, WC-2 and FRQ at the protein level in Figure 7A and at the level of gene regulation of *wc-1* and *frq* in Figure 7B.

### Interdependent regulation of *frq* and *wc-1* by their proteins

The circadian and light input pathways in *Neurospora* interact genetically. Robust *frq* RNA and protein expression depends on WC-1 and WC-2 proteins, which are essential for light responses in *Neurospora* (Figure 2A and B; Crosthwaite *et al.*, 1997). However, *frq* expression is not simply downstream of the WC gene products: we find that basal, DD levels of *wc-1* (Figure 2C) and WC-1 depend on FRQ (Figure 2D; Lee *et al.*, 2000). Since FRQ levels are robustly circadian (Figure 1B; Garceau *et al.*, 1997), one would expect rhythmic *wc-1* RNA in DD, but this is not the case (Figure 1C). A recent report (Lee *et al.*, 2000) also finds that *wc-1* RNA is constitutive but further describes that basal *wc-1* levels are similar in *frq*<sup>+</sup> and *frq*<sup>10</sup>. This is in contrast to what we observe. The most obvious difference in the experimental protocols is the time at which the samples are harvested: after 28 h in darkness in the protocol described here, versus only 6 h in that presented by Lee *et al.* (2000). There could be residual light-induced *wc-1* RNA in *frq*<sup>10</sup> (Figure 4) that persists for at least 6 h in DD; sustainment of those RNA levels may depend on FRQ. WC-1 protein oscillates with a circadian period, out of phase with FRQ (Figure 1D; and as shown in

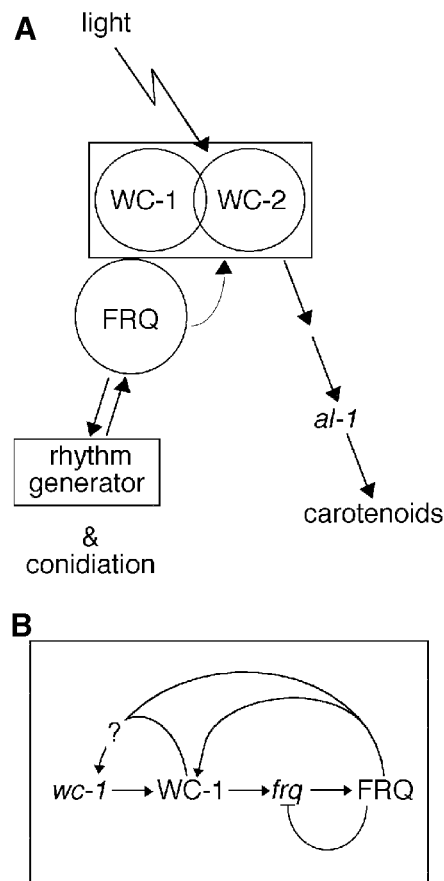


**Fig. 6.** Interaction of FRQ with WC-2 and WC-1. (A) Co-precipitation of FRQ with WC-2 (right panel) and control cell extracts (left panel). For immunoprecipitations, cell extracts were prepared from age-matched mycelial pads grown in DD for the indicated amounts of time. Samples were immunoprecipitated with anti-WC-2 serum (right panel), separated by SDS-PAGE and probed with antibodies against FRQ on western blots. Control cell extracts include those from *wc-2-234w*, *frq<sup>10</sup>* and a strain wild-type at *frq* and *wc-2* loci (*frq<sup>+</sup>*) at DD12 (~CT01) and DD20 (~CT10). These were probed for FRQ and indicate the relative abundance and phosphorylation state of FRQ protein at these times. (B) Co-precipitation of FRQ with WC-1. *frq<sup>+</sup>*, *frq<sup>10</sup>*, *Δwc-1* and *wc-2-234w* were grown in constant light (LL). Extracts were prepared and subjected to immunoprecipitation with antibodies against WC-1. The precipitates were analyzed by SDS-PAGE and blots were probed with antibodies against FRQ. The cell extract of *frq<sup>+</sup>* cultures represents 4% of the input in the co-precipitation experiment and is shown in the right lane as an estimate of the efficiency of the co-precipitation.

Lee *et al.*, 2000), and its regulation includes a post-transcriptional control that is initiated by FRQ expression (Lee *et al.*, 2000). Thus, WC-1 regulates FRQ, and FRQ regulates WC-1, indicating the backbone of the circadian transcription/translation feedback loop in *Neurospora* (see Figure 7B).

### A bifurcated light input pathway

The effect of FRQ on light transduction was investigated by comparing various light-induced responses. Carotenogenesis and conidiation each represent the result of extensive coordination of gene expression and metabolism. Both are regulated by light but, unlike conidiation, carotenogenesis is not circadianly rhythmic in DD. The WCs are essential for both of these light-regulated physiologies (Russo, 1988; Linden *et al.*, 1999), while FRQ is only essential for light-regulated conidiation (Figure 3), indicating a bifurcation in the LIP (Figure 7A). The effect of FRQ on conidiation is specific for light transduction and not for development, *per se*, as conidiation proceeds normally in FRQ-less strains, though without photic or circadian regulation (Figure 3A, tube 3). FRQ has a different function in the branch leading to carotenogenesis: without FRQ, the quantities of light-induced RNAs and carotenoids are lower (Figures 3C and D, and 4).



**Fig. 7.** The molecular components of the *Neurospora* circadian system and the light input pathway (LIP) are inseparable. (A) Our results are compatible with a bifurcation within the LIP, with one branch leading to light-dependent (or, in the absence of light, circadian) conidiation and the other to light-induced carotenogenesis. In terms of regulation by light, WC-1 is required for both branches, while FRQ is only essential for regulated conidiation. Both are important for circadian rhythmicity. In addition, the circadian system modulates the branch regulating carotenoid synthesis, possibly via FRQ. Within the LIP, the WCs and FRQ form a functional unit responsible for both light reception and circadian modulation. (B) Genetic interactions within the *Neurospora* LIP. Although not specified, a basal activation of *wc-1* and *frq* is presumed, in addition to the inferred positive effect on *wc-1* RNA levels by FRQ or WC-1 (see Figure 2). Also, WC-2 is involved in *frq* regulation (Crosthwaite *et al.*, 1997), but this aspect was not addressed in this work.

Additional evidence for a bifurcation in the LIP lies in the fluence responses of the respective branches. While light synchronizes conidiation above a threshold of 2 nE/m<sup>2</sup>/s (gray arrow in Figure 3D; see Merrow *et al.*, 1999; Roenneberg and Merrow, 2001), carotenogenesis is half-maximal at an ~50-fold higher fluence rate (black arrow in Figure 3D).

### Circadian response regulation

FRQ plays a critical role in the *Neurospora* circadian system, as well as in potentiating light-induced carotenogenesis. In addition, we have shown that light responses are stronger at specific circadian times compared with others (Figure 5D). The circadian response regulation is apparent within minutes (e.g. in RNA induction, Figure 5A and C). This represents a general phenomenon whereby evoked sensory processes, which are not rhythmic in constant conditions, are modulated by the circadian

system, e.g. olfaction in *Drosophila* (Krishnan *et al.*, 1999) or chlorophyll production and flowering in plants (Claus and Rau, 1956). In *Neurospora*, the strength of the light-induced response (in the non-circadianly expressed, evoked branch: *wc-1*, *al-1* RNA and carotenogenesis) approximately correlates with the amount of WC-1 protein: no WC-1, no response; low levels of WC-1, weak response; and higher levels of WC-1, stronger response. Thus, FRQ may transduce the circadian modulation of light responses by dictating WC-1 levels (Figure 7A and B).

This correlation, however, is not entirely straightforward. There are some conditions where *Neurospora* is not stimulated by light despite abundant WC-1 protein. During prolonged light exposure, some RNA levels adapt (are down-regulated) although WC-1 levels remain the same (see Figures 4, and 5A and C; Talora *et al.*, 1999). Furthermore, *Neurospora* is refractory to additional light increments for some hours after a dark to light transition (Schmidhauser *et al.*, 1990; Arpaia *et al.*, 1999).

How do FRQ and the WCs functionally participate in the various regulatory processes described above? The WC proteins are thought to possess both photoreceptor and transcription factor activity. FRQ function within the clock has been described with respect to transcriptional control. Indeed, all of these attributes could derive from transcription factor function as modulated by complex formation (Figure 6). Signal transduction by a photoreceptor has been shown to occur via transcriptional control. The plant photoreceptor phytochrome B forms a complex with and modulates the function of a DNA-bound transcription factor (Martinez-Garcia *et al.*, 2000). Interestingly, this particular photoreceptor feeds into the *Arabidopsis* circadian system (Somers *et al.*, 1998).

### FRQ and light entrainment

The response of circadian systems to a given *zeitgeber* signal (e.g. a light pulse) is different at different circadian times. The quantification of these differential responses results in a phase response curve (PRC), a signature for a given circadian system and the respective *zeitgeber*. This quality is the basis for circadian synchronization (entrainment). FRQ is required for the *Neurospora* circadian system to respond to light. Induction of *frq* RNA by short light pulses has been invoked to explain the light PRC of *Neurospora* (Crosthwaite *et al.*, 1995). When *frq* is increased to or above its DD maximum, then the circadian system is reset to the phase of FRQ<sub>max</sub> in DD (Figure 1B). Maximum phase shifts are thus achieved when a light pulse is given at the FRQ minimum (Crosthwaite *et al.*, 1995).

Entrainment is distinct from a driven synchronization whereby a system is turned on or off directly by an exogenous signal. We have shown that light drives conidiation rather than entraining it in a PRC-dependent fashion (Morrow *et al.*, 1999). In LD cycles, conidiation occurs with a constant lag after lights-off, regardless of *zeitgeber* period (i.e. in full photoperiods the response is the same for all tested circadian times). This feature is apparent even at the lowest light fluences that synchronize conidiation (2 nE/m<sup>2</sup>/s, comparable with moonlight, see Figure 3D). In spite of this driven synchronization, the lag between lights-off and conidiation onset is strain specific. It correlates with the period of the specific *frq* allele in DD,

indicating that it depends on the activity of FRQ in the dark phase of the LD cycle. Although light pulse-induced *frq* levels correlate well with phase shifting (Crosthwaite *et al.*, 1995), synchronization of conidiation in full LD cycles appears to function via FRQ protein, independently of its transcription. In the experiments shown in Figure 3A and B (constitutive *frq* expression from the *qa-2* promoter), the lag between lights-off and conidiation is similar to that for *frq*<sup>+</sup> (note that the *qa* promoter *per se* is non-responsive to light; Crosthwaite *et al.*, 1995). One would predict that constitutive expression of different *frq* alleles (similar to the experiments in Figure 3A and B) would also give a strain-specific lag. With regard to mechanism, our experiments show no qualitative or quantitative effects of light on constitutively expressed FRQ protein after 6 or 12 h incubation (Figure 3B); however, acute light effects on FRQ and/or on the formation of the WCC-FRQ complex are possible, and might contribute to the rhythmicity.

Whether circadian systems are entrained primarily by the prolonged presence of light in LD cycles (parametric entrainment) or by acute changes in light (non-parametric entrainment) has long been a point of discussion (Beersma *et al.*, 1999). In *Drosophila*, cryptochrome has been implicated in mediating non-parametric, but not parametric, entrainment (Stanewsky *et al.*, 1998). Our results indicate that synchronization by full photoperiods does not require *frq* transcription, although phase shifting by short light pulses correlates well with *frq* induction (Crosthwaite *et al.*, 1995). Thus, *frq* transcription may be involved in non-parametric light effects, and FRQ protein, together with the WCC complex, may mediate parametric light effects.

So, where does FRQ act within the circadian system: upstream of, central to or downstream of the rhythm generator? Our results show that the participation of FRQ is more complex. Previous results suggest that FRQ is not necessarily central to the rhythm generator (Morrow *et al.*, 1999; Lakin-Thomas and Brody, 2000). Given the fact that conidiation is not regulated by light in its absence, FRQ appears to function as a gateway through which the light signal must pass, placing it upstream of the rhythm generator. Yet without FRQ, light responsiveness (e.g. of carotenogenesis) is not circadianly regulated, placing FRQ downstream of the rhythm generator.

The close association between light input and molecular components of circadian systems is one of the functional parallels characterized from cyanobacteria to mice (Crosthwaite *et al.*, 1997; Shigeyoshi *et al.*, 1997; Emery *et al.*, 1998; Stanewsky *et al.*, 1998; Bognar *et al.*, 1999; Ceriani *et al.*, 1999; Iwasaki *et al.*, 2000; Roenneberg and Morrow, 2000). In addition, the *Neurospora* transcription/translation feedback loop, as described here (and in Lee *et al.*, 2000) shares many features with those of *Drosophila* (Lee *et al.*, 1998; Glossop *et al.*, 1999) and mice (Shearman *et al.*, 2000). In *Neurospora*, it also serves as the LIP.

## Materials and methods

### Strains and media

*frq*<sup>+</sup> and *frq*<sup>0</sup> are standard laboratory strains with the *bd* mutation in their background (Loros *et al.*, 1986; Morrow *et al.*, 1999). *frq*<sup>10</sup> is a knockout of the *frq* locus (Aronson *et al.*, 1994a) and is also on the *bd* background. The  $\Delta wc-1$  strain is a functional knockout, generated by repeat-induced



point (RIP) mutation (Talora *et al.*, 1999), which was crossed with *bd* (*bd Δwc-1* 4–7). RIP is a method for gene inactivation in *Neurospora*, whereby the presence of a duplicate copy of DNA signals methylation and inactivation of both copies when the strain is put through a sexual cross (Selker and Garrett, 1988). This *Δwc-1* strain makes no RNA or protein. *wc-2-234W* is a loss-of-function mutant, which produces a truncated protein (Linden and Macino, 1997), similarly to the *frq<sup>9</sup>* strain (data not shown). *frq<sup>10</sup> qa-2p-frq* describes a strain with the genotype *bd frq<sup>10</sup> his3::his3qa-2p-frqhis*. To generate this, the *bd frq<sup>10</sup> his3* strain was transformed at the *his-3* locus with a *his*-tagged copy of the *frq* open reading frame fused with the *Neurospora qa-2* promoter (for a similar construction see Aronson *et al.*, 1994b).

Race tubes contained 1× Vogel's medium (Vogel, 1964) with 0.5% L-arginine, 10 ng/ml biotin and 2% agar (i.e. no glucose). In some race tubes and liquid cultures (–) quinic acid adjusted to pH 6.0 was added to 10<sup>–3</sup> M. For light induction experiments (Figures 4 and 5A–C), as well as for the experiments shown in Figure 2, stationary, liquid cultures were grown in 1× Vogel's medium with 2% glucose, 0.5% L-arginine and 10 ng/ml biotin. For the circadian time course (Figure 1), the same medium was used but with shaking cultures. For induction of carotenoids (Figure 3C–D and 5D), arginine was omitted from this medium.

#### Race tube experiments and circadian time courses

Race tubes were inoculated, germinated in constant light for ~1 day and transferred to an LD 12:12 cycle at 25°C. The fluence rate was 2 μE/m<sup>2</sup>/s, delivered by cool white fluorescent bulbs (Osram, Germany). Race tubes were analyzed with the Chrono program (Roenneberg and Taylor, 2000). Circadian time courses were performed essentially as described (Aronson *et al.*, 1994b) except for sample intervals (2 h). The time when cultures were transferred to constant darkness is, by definition, CT12 (dusk). Thus in these experiments, the actual time in DD (*t<sub>DD</sub>*) is transformed to CT by the following formula: CT = *t<sub>DD</sub>* × (24/FRP) + 12. For example, for *Neurospora* (*frq<sup>+</sup>*) after 16 h in DD, CT = 16 × (24/22) + 12 = 29.44. By convention, CT is always expressed as 0–24 circadian hours, so for this example, 29.44 – 24 = 5.44.

#### Light-induced mycelial carotenogenesis

Conidia (3 × 10<sup>6</sup>) were inoculated into 20 ml of carotenogenesis medium in 8.5 cm Petri dishes. Sample cultures were evaluated by microscopy for the absence of conidial development. For the experiment shown in Figure 3C, mycelial pads were generated over 2 days incubation at 30°C in constant darkness. For the experiment shown in Figure 3D, pads were cultivated in constant light, then transferred to darkness prior to the dark to light transfers at the indicated times. The transfers to DD were staggered to control for age of the cultures. Excess medium was drained from the dishes for both dark control and light-exposed samples. After 5 h of light exposure, samples were harvested and dried by vacuum filtration. Carotenoid content was determined essentially as described earlier (Linden *et al.*, 1997). A 150 μg aliquot of tissue was disrupted in a Ribolyzer (Hybaid-AGS, Germany) in 500 μl of methanol, 750 μl of hexane, 400 μg of sand and 400 μg of glass beads. The supernatant was collected following centrifugation and the spectral absorption was determined at 445 nm for the experiment shown in Figure 3D. For the experiment shown in Figure 3C, background was subtracted based on the levels of *al-1* mycelial carotenoids, while for Figure 3D the background was determined from the DD levels of mycelial carotenoids in the two strains. For the experiment shown in Figure 5D, because carotenoid induction increases gradually over time in constant darkness, a non-rhythmic trend was subtracted from the time series of carotenoid induction.

#### Light induction experiments

In the experiments shown in Figures 4 and 5, 25 ml of medium in 250 ml flasks was inoculated with 3.5 × 10<sup>6</sup> conidia. These cultures were held in constant light at 30°C for ~1–2 days prior to transfer to constant darkness (DD) at 25°C. Following the indicated time in darkness, age-matched cultures were exposed to continuous light (fluences indicated in the figure legends) until they were harvested by filtration and frozen in liquid nitrogen.

#### RNA analyses

RNA was prepared essentially as previously described (Crosthwaite *et al.*, 1995), except that a single phenol extraction was performed. RNA was run on 1.2% agarose–formaldehyde gels (otherwise as in Merrow *et al.*, 1997), and blotted onto nylon (HybondN, Amersham Corp.). Riboprobes were used to probe the blots for *frq*, *wc-1* and rRNA. The *frq* riboprobe was described elsewhere (Crosthwaite *et al.*, 1995). The

*wc-1* riboprobe was generated from pGEM4wc-1 using T7 RNA polymerase with the linearized plasmid. The riboprobe for rRNA was generated from pEVEII with T3 polymerase, without linearizing. The *al-1* blots were probed and quantified as described (Linden and Macino, 1997). In general, blots were exposed to phosphorimager screens for quantitative analysis. Where films were used for quantification, the GelAnalysis program (T. Roenneberg, University of Munich) was utilized.

#### Protein analyses

Protein was extracted from mycelia by previously described methods (Garceau *et al.*, 1997). In some cases, the tissue was disrupted with the Ribolyzer (Hybaid-AGS, Germany). A 200 μg aliquot per lane was loaded onto 7.5% SDS–polyacrylamide gels for FRQ or 5% gels for WC-1. FRQ blots were probed with the monoclonal antibody mFRQ3G11. Anti-FRQ monoclonal antibodies were produced from C57BL/6×Balb/c F<sub>1</sub> mice immunized with a total of 120 μg of recombinant myelin basic protein (MBP)–FRQ protein in Freund's incomplete adjuvant. Hybridomas were produced using standard techniques and the antibodies were characterized by reactivity against recombinant FRQ in enzyme-linked immunosorbent assay (ELISA) and then on western blots with recombinant deletion proteins and cell extracts from *frq<sup>10</sup>*. The isotype of mFRQ3G11 is IgG1. The antibodies for WC-1 and WC-2 used in Figures 1, 2 and 6A have been described previously (Talora *et al.*, 1999). Co-immunoprecipitations were performed as described (Talora *et al.*, 1999). The specificity of the rabbit anti-WC-1 used in co-immunoprecipitations (Figure 6B) was confirmed with the *Δwc-1* strain.

#### Data analysis

All mathematical fits were carried out by an iterative, least square method. Adaptation profiles were fitted with an equation combining a Gauss distribution (G), a saturated curve (S) and an exponential decay (D):

$$y = G + S + D;$$

$$\text{with } G = a \frac{-(x-b)^2}{2c^2}; S = d \frac{\frac{x}{c}}{\sqrt{1 + \left(\frac{x}{c}\right)^2}}; \text{ and } D = \frac{f}{(x+1)}.$$

All rhythmic time courses were fitted with a cosine function with one exception: due to the non-sinusoidal shape of the time series, WC-1 (Figure 1D) was fitted by eye. The S-curves in Figure 3D were fitted with the following equation:

$$y = a \frac{\frac{(x+b)}{c}}{\sqrt{1 + \left(\frac{x+b}{c}\right)^2}}.$$

#### Acknowledgements

We would like to thank Ina Contag for her help in producing monoclonal antibodies, Vera Schiewe and Astrid Bauer for excellent technical support, and Dr D. Bell-Pedersen for *bd frq<sup>10</sup> his3* strains. This work was supported by a DFG grant to T.R. and the Meyer-Struckmann-Stiftung to M.M. and T.R.; MURST Cofin 99 and Cenci Bolognetti to G.M.; and SFB 455, Project B3 of the DFG to J.J.

#### References

- Aronson, B.D., Johnson, K.A. and Dunlap, J.C. (1994a) The circadian clock locus *frequency*: a single ORF defines period length and temperature compensation. *Proc. Natl Acad. Sci. USA*, **91**, 7683–7687.
- Aronson, B.D., Johnson, K.A., Loros, J.J. and Dunlap, J.C. (1994b) Negative feedback defining a circadian clock: autoregulation of the clock gene *frequency*. *Science*, **263**, 1578–1584.
- Arpaia, G., Loros, J.J., Dunlap, J.C., Morelli, G. and Macino, G. (1993) The interplay of light and the circadian clock. *Plant Physiol.*, **102**, 1299–1305.
- Arpaia, G., Loros, J.J., Dunlap, J.C., Morelli, G. and Macino, G. (1995) Light induction of the clock-controlled gene *cgc-1* is not transduced through the circadian clock in *Neurospora crassa*. *Mol. Gen. Genet.*, **247**, 157–163.

- Arpaia, G., Cerri, F., Baima, S. and Macino, G. (1999) Protein kinase C may be a novel component of the blue light transduction pathway in *Neurospora crassa*. *Mol. Gen. Genet.*, **262**, 314–322.
- Bae, K., Lee, C., Sidote, D., Chuang, K.Y. and Edery, I. (1998) Circadian regulation of a *Drosophila* homolog of the mammalian *Clock* gene: PER and TIM function as positive regulators. *Mol. Cell. Biol.*, **18**, 6142–6151.
- Ballario, P., Vittorioso, P., Magrelli, A., Talora, C., Cabibbo, A. and Macino, G. (1996) *White collar-1*, a central regulator of blue light responses in *Neurospora*, is a zinc finger protein. *EMBO J.*, **15**, 1650–1657.
- Beersma, D.G., Daan, S. and Hut, R.A. (1999) Accuracy of circadian entrainment under fluctuating light conditions: contributions of phase and period responses. *J. Biol. Rhythms*, **14**, 320–329.
- Bognar, L.K., Adam, A.H., Thain, S.C., Nagy, F. and Millar, A.J. (1999) The circadian clock controls the expression pattern of the circadian input photoreceptor, *phytochrome B*. *Proc. Natl Acad. Sci. USA*, **96**, 14652–14657.
- Ceriani, M.F., Darlington, T.K., Staknis, D., Mas, P., Petti, A.A., Weitz, C.J. and Kay, S.A. (1999) Light-dependent sequestration of TIMELESS by CRYPTOCHROME. *Science*, **285**, 553–556.
- Chang, B. and Nakashima, H. (1997) Effects of light–dark cycles on the circadian conidiation rhythm in *Neurospora crassa*. *J. Plant Res.*, **110**, 449–453.
- Claus, H. and Rau, W. (1956) Über die Blütenbildung von *Hyoscyamus niger* und *Arabidopsis* in 72-Stunden-Zyklen. *Z. Bot.*, **44**, 437–454.
- Crosthwaite, S.K., Loros, J.J. and Dunlap, J.C. (1995) Light-induced resetting of a circadian clock is mediated by a rapid increase in *frequency* transcript. *Cell*, **81**, 1003–1012.
- Crosthwaite, S.K., Dunlap, J.C. and Loros, J.J. (1997) *Neurospora wc-1* and *wc-2*: transcription, photoresponses and the origin of circadian rhythmicity. *Science*, **276**, 763–769.
- De Fabo, E.C., Harding, R.W. and Shropshire, W., Jr (1976) Action spectrum between 260 and 800 nanometers for the photoinduction of carotenoid biosynthesis in *Neurospora crassa*. *Plant Physiol.*, **57**, 440–445.
- Degli-Innocenti, F. and Russo, V.E. (1984) Isolation of new *white collar* mutants of *Neurospora crassa* and studies on their behavior in the blue light-induced formation of protoperithecia. *J. Bacteriol.*, **159**, 757–761.
- Dunlap, J.C. (1999) Molecular bases for circadian clocks. *Cell*, **96**, 271–290.
- Emery, P., So, W.V., Kaneko, M., Hall, J.C. and Rosbash, M. (1998) CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell*, **95**, 669–679.
- Feldman, J.F. and Hoyle, M.N. (1973) Isolation of circadian clock mutants of *Neurospora crassa*. *Genetics*, **75**, 605–613.
- Garceau, N.Y., Liu, Y., Loros, J.J. and Dunlap, J. (1997) Alternative initiation of translation and time specific phosphorylation yield multiple forms of essential clock protein FREQUENCY. *Cell*, **89**, 469–476.
- Glossop, N.R.G., Lyons, L.C. and Hardin, P.E. (1999) Interlocked feedback loops within the *Drosophila* circadian oscillator. *Science*, **286**, 766–778.
- Harding, R.W. and Turner, R.V. (1981) Photoregulation of the carotenoid biosynthetic pathway in *albino* and *white collar* mutants of *Neurospora crassa*. *Plant Physiol.*, **68**, 745–749.
- Huala, E., Oeller, P.W., Liscum, E., Han, I.-S., Larsen, E. and Briggs, W.R. (1997) *Arabidopsis* NPH1: a protein kinase with a putative redox-sensing domain. *Science*, **278**, 2120–2123.
- Iwasaki, H., Williams, S.B., Kitayama, Y., Ishiura, M., Golden, S.S. and Kondo, T. (2000) A KaiC-interacting sensory histidine kinase, SasA, necessary to sustain robust circadian oscillation in cyanobacteria. *Cell*, **101**, 223–233.
- Krishnan, B., Dryer, S.E. and Hardin, P.E. (1999) Circadian rhythms in olfactory responses of *Drosophila melanogaster*. *Nature*, **400**, 375–378.
- Lakin-Thomas, P.L. and Brody, S. (2000) Circadian rhythms in *Neurospora crassa*: lipid deficiencies restore robust rhythmicity to null *frequency* and *white-collar* mutants. *Proc. Natl Acad. Sci. USA*, **97**, 256–261.
- Lee, C., Bae, K. and Edery, I. (1998) The *Drosophila* CLOCK protein undergoes daily rhythms in abundance, phosphorylation and interactions with PER–TIM complex. *Neuron*, **21**, 857–867.
- Lee, K., Loros, J.J. and Dunlap, J.C. (2000) Interconnected feedback loops in the *Neurospora* circadian system. *Science*, **289**, 107–110.
- Linden, H. and Macino, G. (1997) *White collar 2*, a partner in blue-light signal transduction controlling expression of light-regulated genes in *Neurospora crassa*. *EMBO J.*, **16**, 98–109.
- Linden, H., Rodriguez-Franco, M. and Macino, G. (1997) Mutants of *Neurospora crassa* defective in regulation of blue light perception. *Mol. Gen. Genet.*, **254**, 111–118.
- Linden, H., Ballario, P., Arpaia, G. and Macino, G. (1999) Seeing the light: news in *Neurospora* blue light signal transduction. *Adv. Genet.*, **41**, 35–54.
- Loros, J.J., Richman, A. and Feldman, J.F. (1986) A recessive circadian clock mutation at the *frq* locus of *Neurospora crassa*. *Genetics*, **114**, 1095–1110.
- Martinez-Garcia, J.F., Huq, E. and Quail, P.H. (2000) Direct targeting of light signals to a promoter element-bound transcription factor. *Science*, **288**, 859–863.
- Marrow, M., Garceau, N. and Dunlap, J. (1997) Dissection of a circadian oscillation into discrete domains. *Proc. Natl Acad. Sci. USA*, **94**, 3877–3882.
- Marrow, M., Brunner, M. and Roenneberg, T. (1999) Assignment of circadian function for the *Neurospora* clock gene *frequency*. *Nature*, **399**, 584–586.
- Millar, A.J., Straumer, M., Chorry, J., Chua, N.-H. and Kay, S.A. (1995) The regulation of circadian period by phototransduction pathways in *Arabidopsis*. *Science*, **267**, 1163–1166.
- Pittendrigh, C.S. (1993) Temporal organization: reflections of a Darwinian clock-watcher. *Annu. Rev. Physiol.*, **55**, 17–54.
- Roenneberg, T. and Deng, T.-S. (1997) Photobiology of the *Gonyaulax* circadian system: I. Different phase response curves for red and blue light. *Planta*, **202**, 494–501.
- Roenneberg, T. and Foster, R.G. (1997) Twilight times—light and the circadian system. *Photochem. Photobiol.*, **66**, 549–561.
- Roenneberg, T. and Mellow, M. (1998) Molecular circadian oscillators—an alternative hypothesis. *J. Biol. Rhythms*, **13**, 167–179.
- Roenneberg, T. and Mellow, M. (1999) Circadian clocks and metabolism. *J. Biol. Rhythms*, **14**, 17–27.
- Roenneberg, T. and Mellow, M. (2000) Circadian light input: omnes viae Romam ducunt. *Curr. Biol.*, **10**, R742–R745.
- Roenneberg, T. and Mellow, M. (2001) The role of feedbacks in circadian systems. In Hiroshige, T. and Honma, K. (eds), *The Brain That Keeps Time*. Hokkaido University Press, Sapporo, in press.
- Roenneberg, T. and Taylor, W. (1994) Light induced phase responses in *Gonyaulax* are drastically altered by creatine. *J. Biol. Rhythms*, **9**, 1–12.
- Roenneberg, T. and Taylor, W. (2000) Automated recordings of bioluminescence with special reference to the analysis of circadian rhythms. *Methods Enzymol.*, **305**, 104–119.
- Russo, V.E. (1988) Blue light induces circadian rhythms in the *bd* mutant of *Neurospora*: double mutants *bd,wc-1* and *bd,wc-2* are blind. *J. Photochem. Photobiol. B*, **2**, 59–65.
- Schmidhauser, T.J., Lauter, F.R., Russo, V.E. and Yanofsky, C. (1990) Cloning, sequence and photoregulation of *al-1*, a carotenoid biosynthetic gene of *Neurospora crassa*. *Mol. Cell. Biol.*, **10**, 5064–5070.
- Selker, E.U. and Garrett, P.W. (1988) DNA sequence duplications trigger gene inactivation in *Neurospora crassa*. *Proc. Natl Acad. Sci. USA*, **85**, 6870–6874.
- Shearman, L.P. et al. (2000) Interacting molecular loops in the mammalian circadian clock. *Science*, **288**, 1013–1019.
- Shigeyoshi, Y. et al. (1997) Light-induced resetting of a mammalian clock is associated with rapid induction of the *mPer1* transcript. *Cell*, **91**, 1043–1053.
- Somers, D.E., Devlin, P.F. and Kay, S.A. (1998) Phytochromes and cryptochromes in the entrainment of the *Arabidopsis* circadian clock. *Science*, **282**, 1488–1490.
- Stanewsky, R., Kaneko, M., Emery, P., Beretta, B., Wagner-Smith, K., Kay, S.A., Rosbash, M. and Hall, J.C. (1998) The *cry<sup>b</sup>* mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*. *Cell*, **95**, 681–692.
- Talora, C., Franchi, L., Linden, H., Ballario, P. and Macino, G. (1999) Role of a white collar-1–white collar-2 complex in blue-light signal transduction. *EMBO J.*, **18**, 4961–4068.
- Vogel, H.J. (1964) Distribution of lysine pathways among fungi: evolutionary implications. *Am. Nat.*, **98**, 435–446.

Received July 13, 2000; revised November 27, 2000;  
accepted November 29, 2000